SPECIFICATION

DNA Encoding Sucrose PTS Enzyme II

Technical Field

The present invention relates to a DNA encoding sucrose PTS enzyme II, which is a protein involved in uptake of sucrose into a cell of coryneform bacterium.

Background Art

Bacteria can assimilate many carbon sources, and various specific systems exist for their cellular transmembrane transport. Moreover, most of bacteria can respond to environmental changes to survive under a limited nutritious condition. Their cells are provided with a detector for monitoring the environment to select their nutrition from various carbon sources. Examples of such transmembrane transport systems and detectors of sugars include PTS (phosphoenolpyruvate/carbohydrate phosphotransferase system or phosphoenolpyruvate-sugar transport system; as for PTS, refer to *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, Second Edition, ASM (American Society for Microbiology) Press).

PTS is involved in regulation of transmembrane transport and phosphorylation of various sugars (PTS sugars), movement towards these carbon sources and many metabolic pathways. PTS catalyzes the following

reaction. PEP refers to phosphoenolpyruvic acid.

PEP (intracellular) + Sugar (extracellular) -->
Pyruvic acid (intracellular) +
Phosphorylated sugar (intracellular)

PTS catalyzes a reaction for generating a phosphorylated sugar and pyruvic acid by translocating a phosphate group of intracellular phosphoenolpyruvic acid (also referred to as "PEP" hereafter) to an extracellular sugar. The phosphorylation of a sugar is linked with cellular transmembrane transport of a sugar, and energies required for these processes are supplied from PEP, which is an intermediate of the glycolytic pathway.

In *Escherichia coli* and *Salmonella typhimurium*, proteins constituting PTS catalyze the following reactions.

(1) PEP + EI --> P-EI + Pyruvic acid

(2) P-EI + Hpr --> P-Hpr + EI

(3) P-Hpr + EIIA --> P-EIIA + Hpr

(4) P-EIIA + EIIB --> P-EIIB + EIIA

(5) P-EIIB + Sugar (extracellular) + EIIB + Sugar-P (intracellular)

Among proteins involved in the above reactions, EI (Enzyme I) and Hpr (histidine protein) are soluble cytoplasmic proteins involved in phosphorylation of all

PTS sugars and referred to as general PTS proteins.

On the other hand, EII (Enzyme II) is specific for PTS sugars and consists of several domains or proteins depending on the sugars. For example, the mannitol-specific EII is a membrane-bound protein consisting of three domains, A, B and C. The glucose-specific EII and sucrose-specific EII consist of IIB and IIC, which are membrane-bound proteins, and IIA, which is a soluble protein. In any case, translocation of a phosphate group from PEP to a sugar is mediated by EI, HPr, EIIA and EIIB. The EIIC domain, which is an intramembranous portion of EII, forms a translocation channel and is considered to be probably a specific binding site of a substrate.

The third type of EII is observed in mannose PTS. Both of its domains A and B are fused in a single soluble polypeptide, and the two intramembranous proteins (IIC and IID) are involved in transmembrane transport of mannose.

In *Escherichia coli* and *Salmonella typhimurium*, the gene encoding EI (ptsI) has been cloned and sequenced (Saffen, E.W. *et al.*, *J. Biol. Chem.*, *262*, pp.16241-16253, 1987; De Reuse, H. and Danchin, A., *J. Bacteriol.*, *170*, pp.3827-3837, 1988). Further, EII specific for some sugars have also been cloned (Saffen, E.W. *et al.*, *J. Biol. Chem.*, *262*, pp.16241-16253, 1987; Erni, B. and Zanolari, B., *J. Biol. Chem.*, *261*,

pp.16398-16403, 1986; Nelson, S.O. *et al.*, *EMBO J.*, *3*, pp.1587-1593, 1984).

It is known that some kinds of sugars are taken up by non-PTS, which do not require PEP, as a system for uptake into cells.

Disclosure of the Invention

As described above, many studies about uptake of sugar into cells have been performed, but studies about PTS in industrially useful coryneform bacteria have not made much progress. Accordingly, an object of the present invention is to provide a gene encoding a protein constituting sucrose PTS in coryneform bacterium.

The inventors of the present application isolated a DNA fragment including a gene encoding sucrase (invertase) of coryneform bacterium and determined its structure. Further, they developed a method for producing an amino acid or a nucleic acid by using a coryneform bacterium containing the amplified sucrase gene (Japanese Patent Laid-open Publication (Kokai) Nos. 5-244958 and 8-196280). In the DNA fragment, four open reading frames (ORF-F1, ORF-F2, ORF-F3 and ORF-F4) exist in a *Sma*I fragment of about 6 kb.

However, the inventors of the present invention considered based on comparison with other sucrase genes that the aforementioned ORF-F2 did not contain the sucrase gene in full length. That is, the number of

amino acid residues in sucrase estimated from known sucrase genes is 466 to 511 (Gunaseakren, P., *J. Bacteriol., 172* (12), pp.6727-35, 1990), whereas the amino acid sequence that can be encoded by ORF-F2 contains 424 amino acid residues, which was relatively short. Therefore, a sequence existing downstream from ORF-F2 was cloned again and its nucleotide sequence was determined. As a result, it was revealed that the DNA fragment containing the aforementioned sucrase gene consisted of two independent cloned fragments ligated to each other, and it was found that a novel gene encoding sucrose PTS enzyme II existed downstream from the sucrase gene. Thus, the present invention was accomplished.

That is, the present invention provides a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 2 in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 2 in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and an activity for binding to sucrose.

The present invention also provides a DNA which encodes a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 2 in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 2 in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and an activity for binding to sucrose.

The aforementioned DNA includes a DNA defined in the following (a) or (b):

(a) a DNA which contains the nucleotide sequence of the nucleotides 3779 to 5761 of SEQ ID NO: 1 in Sequence Listing;

(b) a DNA which is hybridizable with a nucleotide sequence containing the nucleotide sequence of the nucleotides 3779 to 5761 of SEQ ID NO: 1 in Sequence Listing under a stringent condition, and encodes a protein having an activity for binding to sucrose.

Brief Description of the Drawings

Fig. 1 shows a construction process of a plasmid for disrupting the sucrose PTS enzyme II gene.

Fig. 2 shows a construction process of pBCT4.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained in detail.

The DNA of the present invention was obtained, in the examples described later, by amplifying a region existing downstream from the sucrase gene on chromosomal

DNA of *Brevibacterium lactofermentum* by PCR (polymerase chain reaction).

A region adjacent to a known region on a chromosomal DNA can be amplified by ligating a cassette to a DNA fragment containing the regions and performing PCR using a primer corresponding to the known region and a primer corresponding to the cassette. At this time, if the 5' end of the cassette is dephosphorylated beforehand, a nick is generated at a ligation site of the chromosomal DNA fragment and 5' end of the cassette. Therefore, DNA synthesis started from the cassette primer will be stopped at this ligation site, and only the DNA synthesized from a synthetic primer will serves as a template for synthesis starting from the cassette primer and a complementary chain will be formed. As a result, specific amplification becomes possible (cassette-ligation mediated PCR method (*Molecular and Cellular Probes, 6,* pp.467-475)). A kit utilizing this method is commercially available (TAKARA LA PCR™ in vitro Cloning Kit, Takara Shuzo) and can be utilized to obtain the DNA of the present invention.

Since the nucleotide sequences of the DNA of the present invention and the adjacent region thereof have been revealed, they can be directly amplified by PCR using oligonucleotides synthesized based on these nucleotide sequences as primers and chromosomal DNA of coryneform bacterium as a template. Examples of such

primers include oligonucleotides having the nucleotide sequences of SEQ ID NOS: 10 and 21. Further, the DNA of the present invention can also be isolated from a chromosomal DNA library by hybridization using an oligonucleotide synthesized based on these nucleotide sequences as a probe. The chromosomal DNA of coryneform bacterium can be obtained by, for example, the method of Saito *et al.* (described in *Biochim. Biophys. Acta, 72,* pp.619-629, 1963) or the method of K. S. Kirby (*Biochem. J., 64,* p.405, 1956).

Further, conventional methods well known to those skilled in the art can be employed for preparation of chromosomal DNA, preparation of chromosomal DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation, design of oligonucleotides used as primers and so forth. These methods are described in Sambrook, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, 1989 and so forth.

Plasmids used for cloning of the DNA of the present invention, preparation of a chromosomal DNA library or the like may be those that can replicate in microorganisms such as bacteria belonging to the genus *Escherichia*, and specific examples thereof include pBR322, pTWV228, pMW119, pUC19 and so forth.

An example of the nucleotide sequence of a DNA

fragment containing the DNA of the present invention obtained as described above is shown as SEQ ID NO: 1 in Sequence Listing. The region containing the nucleotide sequence of the nucleotides 3779 to 5761 in this nucleotide sequence encodes sucrose PTS enzyme II, which is the protein of the present invention. In the nucleotide sequence of SEQ ID NO: 1, the nucleotides 342 to 1505 and the nucleotides 2338 to 3609 correspond to ORF-F1 and ORF-F2, respectively, in the DNA fragment containing the sucrase gene described in Japanese Patent Laid-open Publication (Kokai) No. 8-196280. Further, when the nucleotide sequence of SEQ ID NO: 1 and the nucleotide sequence described in Japanese Patent Laid-open Publication (Kokai) No. 8-196280 are compared, a region of the nucleotides 1 to 3687 in the nucleotide sequence of SEQ ID NO: 1 was identical to the nucleotide sequence described in Japanese Patent Laid-open Publication (Kokai) No. 8-196280. Accordingly, it was revealed that the DNA fragment containing the sucrase gene consisted of two independent cloned fragments.

The DNA of the present invention may be a DNA that encodes sucrose PTS enzyme II including substitution, deletion, insertion, addition or inversion of one or several amino acids at one or a plurality of sites so long as the activity for binding to sucrose of the encoded sucrose PTS enzyme II is not deteriorated. The number meant by the term "several" used herein may vary

depending on locations of amino acid residues in the three-dimensional structure of proteins and kinds of amino acid residues. This is due to the fact that there are highly analogous amino acids among amino acids such as isoleucine and valine, and difference among such amino acids does not substantially affect the three-dimensional structure of proteins. Therefore, the protein may be one having homology of 70% to 80% or higher, preferably, 90% to 95%, with respect to the whole amino acid sequence constituting sucrose PTS enzyme II and having an activity for binding to sucrose. Specifically, the term "several" means 2 to 180, preferably 2 to 60, more preferably 2 to 5.

Such a DNA encoding a protein which is substantially identical to sucrose PTS enzyme II as mentioned above can be obtained by modifying a nucleotide sequence so that the amino acid sequence at a particular site should include substitution, deletion, insertion, addition or inversion of an amino acid residue or residues through, for example, site-specific mutagenesis. Further, such a modified DNA as mentioned above may also be obtained by a conventional mutagenesis treatment. Examples of the mutagenesis treatment include an in vitro treatment of DNA encoding sucrose PTS enzyme II with hydroxylamine or the like, a treatment of microorganisms such as *Escherichia* bacteria containing the DNA encoding sucrose PTS enzyme II by UV

irradiation or with mutagenesis agents used for a usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid.

The aforementioned substitution, deletion, insertion, addition, inversion or the like of nucleotides includes naturally occurring mutations (mutant or variant) such as those observed depending on differences of strains, species or genera of microorganisms containing sucrose PTS enzyme II and so forth.

A DNA which encodes a protein substantially the same as sucrose PTS enzyme II can be obtained by, for example, isolating a DNA which is hybridizable with a DNA having the nucleotide sequence of the nucleotides 3779 to 5761 of the nucleotide sequence of SEQ ID NO: 1 in Sequence Listing or a probe prepared from DNA having the nucleotide sequence by PCR or the like under stringent conditions and encoding a protein containing sucrose PTS enzyme II having an activity for binding to sucrose from DNA encoding sucrose PTS enzyme II containing a mutation or a cell containing it. The "stringent conditions" referred to herein is a condition under which a so-called specific hybrid is formed, but a non-specific hybrid is not formed. It is difficult to clearly define this condition by using numerical values. However, for example, the stringent conditions include a condition under which two of DNAs having high homology,

for example, two of DNAs having homology of not less than 50% are hybridized with each other, but two of DNAs having homology lower than the above level are not hybridized with each other. Alternatively, the stringent conditions are exemplified by a hybridization condition represented by salt concentrations of 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C, which is an ordinary condition of washing in Southern hybridization. The homology used herein is represented with a value calculated by the method of Lipman-Pearson (*Science*, *227*, pp.1435-1441, 1985) or the method of Takashi & Gotoh (*J. Biochem.*, *92*, pp.1173-1177, 1984). The probe can be designed according to a method known to those skilled in the art.

Those genes hybridizable under the condition as described above include those having a stop codon generated in the genes, but such genes can be easily removed by ligating them to a commercially available expression vector to examine size of the expressed product.

The protein of the present invention is a protein encoded by the DNA of the present invention and has the amino acid sequence of SEQ ID NO: 2. The protein of the present invention may have an amino acid sequence corresponding to the amino acid sequence of SEQ ID NO: 2 in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino

acids so long as it has an activity for binding to sucrose.

The DNA of the present invention can be utilized to improve sucrose uptake ability of coryneform bacteria or the like. Further, since PTS consumes PEP for uptake of a sugar into a cell, PTS is considered to be disadvantageous for synthesis of amino acids of which biosynthesis system include PEP in an upstream stage. Therefore, if sucrose PTS is disrupted and sucrose can be taken up by an uptake system which does not require PEP, it is considered advantageous in view of sucrose uptake rate or productivity of an amino acid or the like. In coryneform bacteria, non-PTS specific for sucrose is not known, but, for example, if sucrase is allowed to act extracellularly, glucose and fructose can be taken up by non-PTS.

Further, if the DNA of the present invention is modified so as to encode sucrose PTS enzyme II having an enhanced or suppressed function or so as to be ligated to an expression control sequence such as a promoter derived from other genes and introduced into a coryneform bacterium, a coryneform bacterium having an enhanced or suppressed sucrose uptake ability can be created. Specifically, a DNA encoding sucrose PTS enzyme II having an enhanced function is introduced into an autonomously replicable vector or chromosomal DNA in a cell of coryneform bacterium. Further, a DNA encoding

sucrose PTS enzyme II having a suppressed function is introduced into chromosomal DNA by gene substitution utilizing homologous recombination. Alternatively, a coryneform bacterium in which sucrose PTS functions at low temperature but does not function at high temperature can be created by gene substitution using a plasmid containing a temperature sensitive replication control region (see Japanese Patent Publication (Kokoku) No. 7-108228).

Coryneform bacteria to which the present invention is applicable include those bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, *41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are mentioned below.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

Corynebacterium herculis

Brevibacterium divaricatum (*Corynebacterium glutamicum*)

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium immariophilum

Brevibacterium lactofermentum (*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium ammoniagenes (*Corynebacterium ammoniagenes*)

Brevibacterium album

Brevibacterium cerium

Microbacterium ammoniaphilum

Examples of the vector autonomously replicable in a cell of coryneform bacterium include pAM330 (refer to Japanese Patent Laid-open (Kokai) No. 58-67699), pHM1519 (refer to Japanese Patent Laid-open (Kokai) No. 58-77895) and so forth. Moreover, if a DNA fragment having an ability to make a plasmid autonomously replicable in coryneform bacterium is excised from these vectors and inserted into the vectors for *Escherichia coli*, they can be used as a so-called shuttle vector autonomously replicable in both of *Escherichia coli* and coryneform bacteria. Examples of such a shuttle vector include those mentioned below. There are also indicated microorganisms that harbor each vector, and accession

numbers thereof at international depositories are shown in the parentheses, respectively. Among these, pHSC4 includes a temperature sensitive replication control region.

pAJ655	*Escherichia coli* AJ11882 (FERM BP-136)
	Corynebacterium glutamicum SR8201 (ATCC39135)
pAJ1844	*Escherichia coli* AJ11883 (FERM BP-137)
	Corynebacterium glutamicum SR8202 (ATCC39136)
pAJ611	*Escherichia coli* AJ11884 (FERM BP-138)
pAJ3148	*Corynebacterium glutamicum* SR8203 (ATCC39137)
pAJ440	*Bacillus subtilis* AJ11901 (FERM BP-140)
pHC4	*Escherichia coli* AJ12617 (FERM BP-3532)
pHSC4	*Escherichia coli* AJ12571 (FERM BP-3524)

A recombinant vector containing the DNA of the present invention can be introduced into a coryneform bacterium according to a transformation method reported so far. For instance, there are a method of treating recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for *Escherichia coli* K-12 (Mandel, M. and Higa, A., *J. Mol. Biol., 53,* 159, 1970); and a method of preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene, 1,* 153, 1977). In

addition to these, also employable are a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing the recombinant DNA into the DNA-recipient cells, which method is known to be applicable to *Bacillus subtilis*, actinomycetes and yeasts (Chang, S. and Choen, S.N., *Molec. Gen. Genet.*, *168*, 111, 1979; Bibb, M.J., Ward, J.M. and Hopwood, O.A., *Nature*, *274*, 398, 1978; Hinnen, A., Hicks, J.B. and Fink, G.R., *Proc. Natl. Acad. Sci., USA*, *75*, 1929, 1978) and the electric pulse method (see Japanese Patent Laid-open Publication (Kokai) No. 2-207791).

Examples

Hereafter, examples of the present invention will be explained in detail.

Example 1: Isolation of gene encoding sucrose PTS enzyme II

<1> Analysis of chromosomal DNA of *Brevibacterium lactofermentum* AJ12036 (FERM BP-734) by Southern hybridization

The *Brevibacterium lactofermentum* AJ12036 strain was cultured overnight in 4 ml of M-CM2S medium (containing 5 g/L of sucrose, 10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl and 0.1 g/L of DL-methionine) and microbial cells were collected.

Chromosomal DNA was extracted from the obtained microbial cells by using a Bacterial Geneomic DNA Purification Kit (Advanced Genetic Technologies). The chromosomal DNA was eluted with 50 µl of TE buffer (composition: 10 mM tris-HCl (pH 7.5), 1 mM EDTA-2Na).

The chromosomal DNA extracted as described above was subjected to Southern hybridization according to the method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The chromosomal DNA was separately digested with *Bam*HI and *Sma*I, which did not cleave regions on the C-terminus side of ORF-F2 and N-terminus side of ORF-F3 and subjected to agarose gel electrophoresis. As a probe, a fragment of about 3 kb was used that was excised from 6.9 kb fragment cloned on pSSM30 (Japanese Patent Laid-open Publication (Kokai) No. 8-196280) with *Bam*HI to cover the regions on the C-terminus side of ORF-F2 and on the N-terminus side of ORF-F3 (Japanese Patent Laid-open Publication (Kokai) No. 8-196280, the fragment of SEQ ID NO: 1649 to 4675 in Sequence Listing).

As a result of the hybridization, two bands were detected, and it was revealed that ORF-F2 and ORF-F3 were not adjacent to each other on the chromosome. Therefore, it was attempted to confirm a sequence existing downstream from the sucrase gene again.

<2> Determination of sequence of region existing downstream from sucrase gene

To determine the nucleotide sequence of a region downstream from the sucrase gene, the downstream region was first amplified by PCR. PCR was performed by using a TAKARA LA PCR™ in vitro Cloning Kit (Takara Shuzo). Specifically, the PCR was performed as follows.

The chromosomal DNA was completely digested with 10 kinds of restriction enzymes (*Spe*I, *Eco*T14I, *Nhe*I, *Pst*I, *Eco*T22I, *Bgl*II, *Bam*HI, *Xho*I, *Sal*I, *Ava*I), which produced the same cleavage ends as cassettes (SEQ ID NOS: 3 to 8 in Sequence Listing) attached to the aforementioned kit. PCR was performed by using each of these fragments as a template, Synthetic primer 1 shown in Table 1 and Cassette primer 1 (SEQ ID NO: 19). Since a phosphate group was not added to the 5' end of the cassette, a nick was generated at the ligation site of the chromosomal DNA fragment and the 5' end of the cassette. Therefore, the DNA synthesis starting from the cassette primer stopped at this ligation site, and only the DNA synthesized from the synthetic primer served as a template for synthesis starting from the cassette primer, and a complementary chain was formed.

Subsequently, PCR was performed out by using the amplification product obtained above as a template, Synthetic primer 2 and Cassette primer 2 (SEQ ID NO: 20). As a result, a fragment could be amplified when a DNA

obtained by digesting the chromosomal DNA with *Eco*T14I, *Pst*I, *Bgl*II, *Bam*HI, *Xho*I or *Ava*I was used as a template. The nucleotide sequence of a fragment of about 1.8 kb amplified by using the DNA fragment digested with *Bam*HI as a template was determined.

Table 1: Nucleotide sequence and position of synthetic primer

Primer number	Nucleotide sequence	Location in SEQ ID NO: 1 (nucleotide number)
1	CGTCTTGCGAGGATTCAGCGAGCTG (SEQ ID NO: 9)	(3159 to 3183)
2	AGCTGGATTTCGGCCATGAATTCTA (SEQ ID NO: 10)	(3179 to 3203)
3	GATCTGTTCGGTCCGCAATCACT (SEQ ID NO: 11)	(4189 to 4212)
4	CACTGGTGGAGATGTTCCCTCAGAT (SEQ ID NO: 12)	(4209 to 4233)
5	CATCTTCGCAACCGCATCCATGGCC (SEQ ID NO: 13)	(4801 to 4825)
6	CGCGCAGGGTGCAGCATGTTTGGC (SEQ ID NO: 14)	(4831 to 4854)
7	GGGCCTTGCAGGTGCTTCAGGTGTC (SEQ ID NO: 15)	(4888 to 4912)
8	CCGCTGTTCTTGGTATTACAGAGCC (SEQ ID NO: 16)	(4914 to 4938)
9	GCAGCGTCAGCGATGCCATGTTTGC (SEQ ID NO: 17)	(5322 to 5346)
10	GCTTGGCTCAGGTGTTGCGATCGTC (SEQ ID NO: 18)	(5356 to 5380)

Synthetic primers 3 and 4 were synthesized based on the determined sequences. In the same manner as described above, the fragments were successively amplified by PCR using a combination of Synthetic primer 3 and Cassette primer 1 and a combination of Synthetic

primer 4 and Cassette primer 2. As a result, a fragment could be amplified when a DNA obtained by digesting the chromosomal DNA with *Pst*I or *Bam*HI was used as a template. The nucleotide sequence of the fragment amplified based on the DNA fragment digested with *Pst*I was determined.

Synthetic primers 5 and 6 were synthesized based on the determined sequence. PCR was successively carried out by using a combination of Synthetic primer 5 and Cassette primer 1 and a combination of Synthetic primer 6 and Cassette primer 2. As a result, an amplified fragment could be confirmed when the chromosomal DNA digested with *Eco*T14 or *Pst*I was used as a template. The nucleotide sequence of the fragment of the former case was determined.

Further, Synthetic primers 7 and 8 were synthesized and the same procedure as described above was performed. As a result, an amplified fragment could be confirmed when *Eco*T14-digested chromosomal DNA was used as a template. The nucleotide sequence of this amplified fragment was determined.

Primers 9 and 10 were synthesized based on the above sequence, and the same procedure as described above was performed. As a result, an amplified fragment could be confirmed when *Spe*I-digested chromosomal DNA was used as a template. The nucleotide sequence of this amplified fragment was determined.

As for the nucleotide sequence determination, a reaction was performed by using a sequencing kit produced by ABI according to its protocol, and then the nucleotide sequence of the amplified fragment was determined by the fluorescence labeling method.

The above results are shown in SEQ ID NO: 1 in Sequence Listing. It was found that a novel ORF existed after the nucleotide number 3684 in this nucleotide sequence. It was inferred that this ORF consisted of the nucleotide sequence of 1983 bp corresponding to the nucleotide numbers 3779 to 5761, and that a protein obtained by translating the determined nucleotide sequence consisted of 661 amino acids. As for the ORF, homology search was performed in the GENBANK CDS database. As a result, as shown in Table 2, the proteins that could be encoded by the ORF showed high homology with sucrose PTS enzyme II, a protein specific for sucrose uptake. Hereafter, this ORF is referred to as ptsIIsuc gene.

Table 2: Results of homology search of novel ORF

Name of Bacterium and gene		Known protein showing homology	Homology (%)
P. pentsaceus	scrA	Enzyme IIscr	48.8
B. subtilis	treP	Trehalose-specific enzyme IIBC	43.4
S. xylosus	scrA	Enzyme IIscr	52.2
S. mutans	scrA	Enzyme IIscr	45.4
S. typhimurium plasmid pUR400	scrA	Enzyme Iiscr	37.6

Example 2: Preparation of sucrose PTS enzyme II gene-disrupted strain

A *Brevibacterium lactofermentum* strain with a disrupted ptsIIsuc gene was prepared. First of all, a plasmid for disrupting the gene was prepared (Fig. 1). A ptsIIsuc gene fragment amplified by PCR using the chromosome of *Brevibacterium lactofermentum* AJ12036 as a template, Primer 2 (SEQ ID NO: 10) and Primer 11 (SEQ ID NO: 21) having the nucleotide sequence shown below was cloned by using a TA cloning kit (Invitrogen), and the plasmid was designated as pCRS2.

(Primer 11)

CGCTACTGCTGAACGAACATGTCC (corresponding to the nucleotide numbers 5947 to 5924 in SEQ ID NO: 1)

A fragment excised from pCRS2 by digestion with *Xba*I and *Spe*I was ligated to the *Xba*I ends of pHSG399 to construct p399S2. This plasmid was digested with *Hpa*I and *Bam*HI, and the obtained fragment (corresponding to

the nucleotide numbers 4385 to 4798 in SEQ ID NO: 1) was ligated to pHSG299 digested with *Sma*I and *Bam*HI to prepare a plasmid pdSB. Subsequently, pdSB was digested with *Bam*HI and ligated to a temperature sensitive replication origin that was excised from plasmid pBCT4 by digestion with *Bam*HI and could replicate in coryneform bacteria (refer to Japanese Patent Publication (Kokoku) No. 7-108228) to prepare a plasmid pdSBT. The plasmid included the ptsIIsuc gene having deleted 5'end and 3' ends. The pdSBT could autonomously replicate in coryneform bacteria at about 10°C to 32°C, but not at about 34°C or higher.

The pBCT4 was constructed as follows. A temperature sensitive vector pHSC4 described in Japanese Patent Publication (Kokoku) No. 7-108228 was digested with restriction enzymes, *Bam*HI and *Kpn*I, to obtain a DNA fragment of about 3 kb containing the obtained temperature sensitive replication origin. Both of the ends of the obtained DNA fragment were blunt-ended with T4 DNA polymerase. This DNA fragment was ligated with *Bam*HI linkers and digested with *Bam*HI again. Then, it was ligated to pHSG399 digested with *Bam*HI to obtain pBCT4 (Fig. 2).

The *Brevibacterium lactofermentum* AJ12036 strain was transformed with pdSBT and a transformant was selected by using a CM2S plate containing 25 μg/ml of kanamycin. The transformation was performed by the

electric pulse method (refer to Japanese Patent Laid-open Publication (Kokai) No. 2-207791). The obtained transformant was designated as AJ12036/pTSBT. The AJ12036/pTSBT strain was diluted and spread on M-CM2S plates containing 25 μg/ml of kanamycin at 10^3 to 10^5 cfu per plate. The transformants on the plates were cultured overnight at 34°C, and a strain showing drug resistance was obtained as a strain containing the plasmid incorporated into its chromosome. It was confirmed by PCR that the obtained strain had the vector plasmid incorporated into the pTSIIsuc gene of the host chromosome by homologous recombination. This integrated strain was designated as YdS1.

The YdS1 strain was cultured at 34°C in a minimal medium containing glucose or sucrose as a sugar source (20g/L of glucose or sucrose, 5 g/L of ammonium sulfate, 2 g/L of urea, 1 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.002 g/dl of $FeSO_4$, 0.002 g/dl of $MnSO_4$, 100 μg/L of biotin, 2000 μg/L of vitamin B1, 10 mg/dl of DL-methionine and 15 g/L of agar, pH 6.6). The results are shown in Table 3. Since the YdS1 strain could grow in the minimal medium containing only glucose as a carbon source, but not in the minimal medium containing only sucrose as a carbon source, it was confirmed that the ptsIIsuc gene is the gene encoding Enzyme II which is a protein specific for sucrose in sucrose uptake.

Table 3: Growth on minimal medium

Bacterial strain	Carbon source	
	Sucrose	Glucose
AJ12036	Possible to grow	Possible to grow
YdS1	Impossible to grow	Possible to grow

Industrial Applicability

The present invention provides a gene encoding sucrose PTS enzyme II of coryneform bacterium and a strain of coryneform bacterium in which sucrose PTS does not function. These gene and bacterial strain can be utilized in breeding of strains with improved sugar uptake rate or improved productivity of an amino acid, a nucleic acid or the like.